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# *In Vitro* Kinetic Study of the Squalestatin Tetraketide Synthase Dehydratase Reveals the Stereochemical Course of a Fungal Highly Reducing Polyketide Synthase

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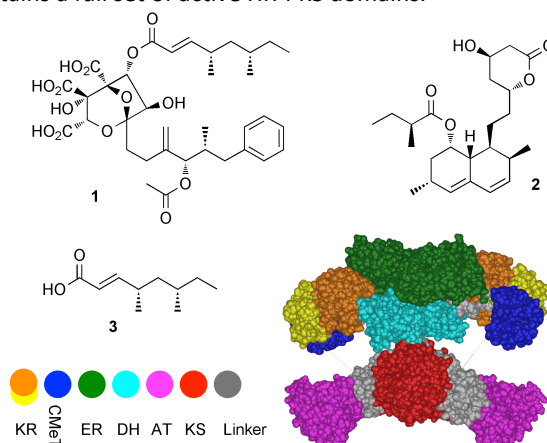
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Six potential diketide substrates for the squalestatin tetraketide synthase (SQTKS) dehydratase (DH) domain were synthesised as *N*-acetyl cysteamine thioesters (SNAC) and tested in kinetic assays as substrates with an isolated DH domain. 3*R*-3-Hydroxybutyryl SNAC 3*R*-16 was turned over by the enzyme, but its enantiomer was not. Of the four 2-methyl substrates only 2*R*,3*R*-2-methyl-3-hydroxybutyryl SNAC 2*R*,3*R*-8 was a substrate. Combined with stereochemical information from the isolated SQTKS enoyl reductase (ER) domain, our results provide a near complete stereochemical description of the first cycle of beta-modification reactions of a fungal highly reducing polyketide synthase (HR-PKS). The results emphasise the close relationship between fungal HR-PKS and vertebrate fatty acid synthases (vFAS).

Iterative fungal polyketide synthases (PKS) are responsible for the biosynthesis of complex and often biologically active natural products such as squalestatin S1 **1** a potent inhibitor of squalene synthase,<sup>1,2</sup> and lovastatin **2**,<sup>3</sup> an inhibitor of human HMG-CoA reductase. These PKS are Type I systems in which several individual catalytic domains are covalently linked to form a mega-complex of *ca* >200 kDa.<sup>4,5</sup> Understanding the selectivity and programming of these systems is important because reprogramming them could lead to the systematic creation of new bioactive materials. In order to achieve this an understanding of the individual catalytic domains and their intrinsic selectivities is required.

The C<sub>10</sub> side-chain of **1**, known as squalestatin tetraketide **3**, is synthesised by a highly reducing (HR) iterative PKS called squalestatin tetraketide synthase (SQTKS).<sup>6,7</sup> It consists of an acyl carrier protein (ACP) which holds the growing polyketide chain, a  $\beta$ -ketoacyl ACP synthase (KS) which catalyses a Claisen condensation between malonyl ACP and acyl-KS and an acyl transferase (AT) which loads acetyl starter and malonyl

extender units from CoA onto the PKS. In addition, SQTKS contains:  $\beta$ -ketoacyl ACP ketoreductase (KR);  $\beta$ -hydroxy acyl ACP dehydratase (DH); and enoyl ACP reductase (ER) domains which process the  $\beta$ -carbonyl formed by the KS. Finally, a C-methyl transferase (C-MeT) domain is responsible for adding a methyl group derived from *S*-adenosyl methionine. SQTKS thus contains a full set of active HR-PKS domains.



**Figure 1.** Structures of fungal highly reduced polyketides, and the structure of dimeric vFAS (PDB 2VZ8)<sup>8</sup> which is homologous the squalestatin tetraketide synthase (SQTKS). See text for abbreviations.

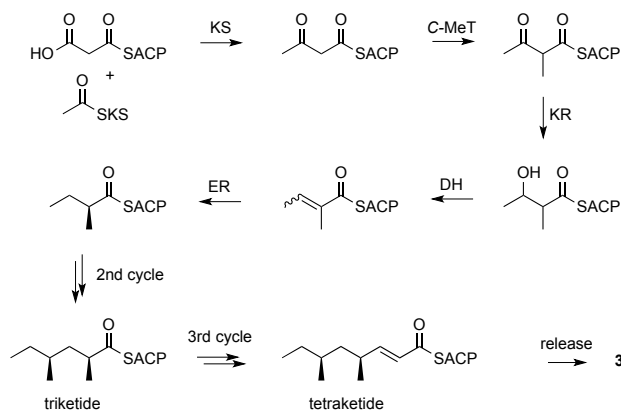
SQTKS shows sequence homology to vertebrate FAS (vFAS, Figure 1).<sup>8</sup> This similarity even extends to the position of a C-MeT domain which is inactive in vFAS, but which acts during the first and second rounds of chain processing by SQTKS.<sup>9</sup> vFAS produces fully saturated linear 16–18 carbon chains, whereas SQTKS produces the dimethylated and unsaturated 8-carbon chain **3**. SQTKS thus displays a complex programme in which the activities of the individual catalytic domains can be varied (Scheme 1). Our approach to study the programming mechanisms of fungal HR-PKS is to examine intrinsic selectivities of isolated catalytic domains. For example, we recently reported on the chemo- and stereo-selectivity of the isolated SQTKS ER domain.<sup>10</sup> Here we describe work to extend this study to the isolated DH domain of SQTKS.

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**Scheme 1.** The chemical reactions catalysed by SQTKS.

SQTKS is a megacomplex of 284.4 kDa encoded by the *phpks1* gene. We have been unable to obtain it as a single soluble protein. However, by systematic variation of possible start and stop positions for PCR from an intron-free *phpks1* template, we were able to create an open reading frame which reliably produces soluble SQTKS DH protein when expressed in *E. coli* BL21 with an N-terminal his<sub>6</sub> tag. The DH protein of the expected 38.0 kDa was purified to homogeneity by nickel affinity and gel-filtration chromatography. Calibrated gel filtration indicated that the DH exists primarily as a monomer (see ESI). The isolated DH was unstable in unmodified buffers, precipitating rapidly even at low temperatures. However, rapid removal of imidazole used for the nickel ion chromatography and use of a buffer containing 10% glycerol, 50 mM Tris pH 8.0, 150 mM NaCl and 100 mM L-arginine and L-glutamic acid dramatically improved protein solubility and stability.

N-acetyl cysteamine (NAC) is a truncated form of the phosphopantetheine (PP) cofactor which attaches acyl PKS intermediates to the ACP domain, and SNAC thioesters are often used as PP surrogates for *in vitro* studies of PKS enzymes,<sup>11,12</sup> including DH domains.<sup>13,14</sup> We thus selected SNACs as targets for substrate synthesis (Scheme 2).

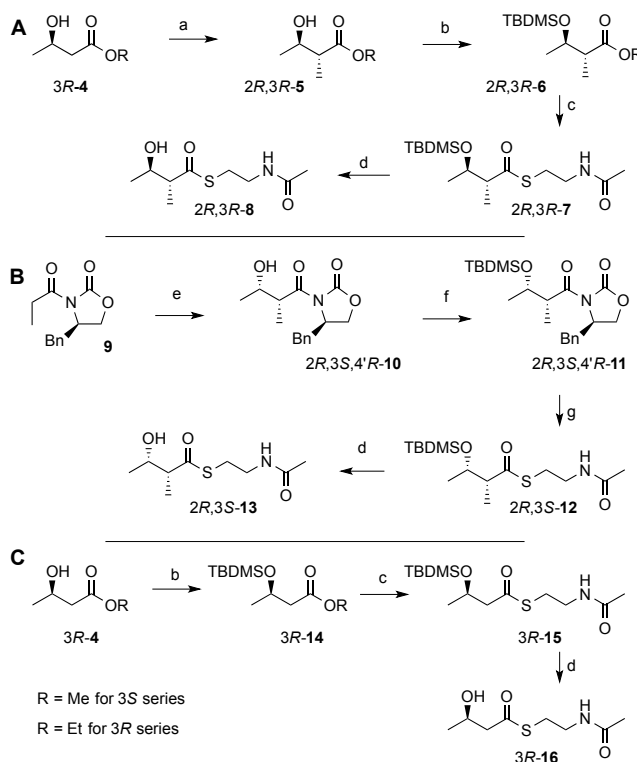
The *anti* diketide SNAC **2R,3R-8** was made by a route involving Fráter-Seebach methylation<sup>15</sup> of commercially available enantiopure 3-hydroxy butyrate **4** (Scheme 2A) to give **5**. This was *O*-protected with TBDMS to give **6**, which was in-turn hydrolysed to its corresponding acid and coupled to HSNAC to give the protected diketide **7**. Acidic deprotection then yielded **2R,3R-8**.

The *syn* diketide SNAC **2R,3S-13** was made using Evans asymmetric aldol chemistry<sup>16</sup> to give the known *syn* aldol product **2R,3S,4'R-10** (Scheme 2B). This was again *O*-protected with TBDMS to give **11**, which was hydrolysed and processed to the protected SNAC **2R,3S-12**. Acidic deprotection then yielded **2R,3S-13**.

The non-methylated diketide **3R-16** was made from **4** by a similar protection, thioesterification and deprotection route (Scheme 2C). The enantiomers of all the diketides were made from enantiomeric starting materials using identical methods.

DH activity was assayed using LCMS (See ESI) to measure substrate consumption and product formation. Assays were

set up to include DH protein, substrate and buffer at 30 °C in 100 µL assay volume, and 20 µL aliquots were taken at time points and quenched in CH<sub>3</sub>CN (60 µL). Protein was precipitated by centrifugation and the supernatant was examined directly by LCMS.

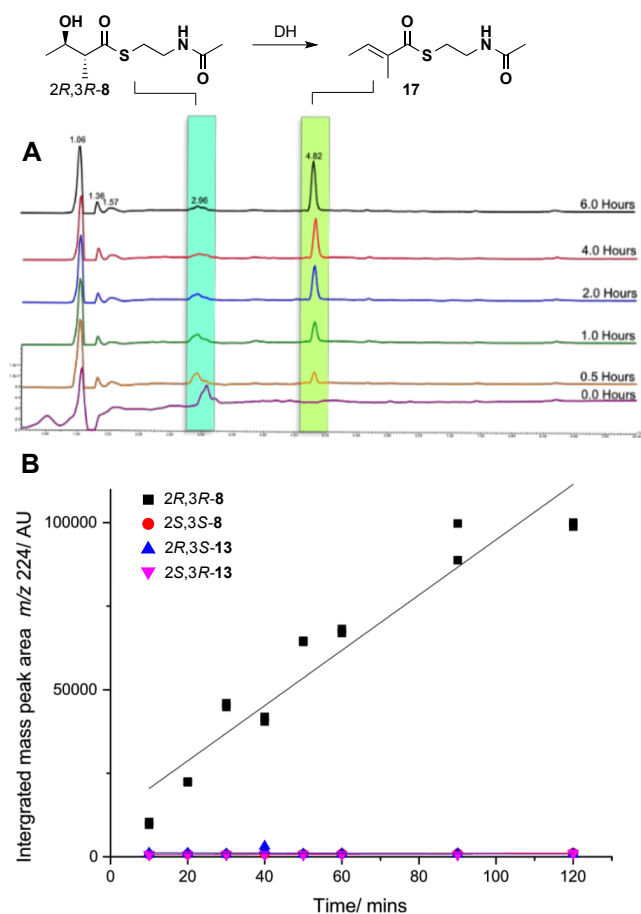


**Scheme 2.** Synthesis of potential SNAC substrates for the SQTKS DH domain: **a**, LDA (2eq.), -78 °C, then MeI; **b**, TBDMSOTf, pyridine, 0 °C; **c**, aq. LiOH, 60 °C, then HSNAC, EDCI, DMAP, 0 °C; **d**, THF/H<sub>2</sub>O/HOAc, RT, 5 days; **e**, Bu<sub>2</sub>BOTf, CH<sub>2</sub>Cl<sub>2</sub>, Et<sub>3</sub>N, -78 °C, then CH<sub>3</sub>CHO; **f**, TBDMSOTf, CH<sub>2</sub>Cl<sub>2</sub>, DMAP, imidazole, RT; **g**, LiOH, H<sub>2</sub>O, RT, then HSNAC, EDCI, DMAP, 0 °C. TBDMS = Si(Me)<sub>2</sub>CMMe<sub>3</sub>.

In order to maximise sensitivity, single ion monitoring was applied for substrate and product peaks and peak areas were integrated. The peak integrals were calibrated vs known concentrations of substrate and product. Initial rates were determined by plotting product concentration vs assay times, and variation of initial substrate concentrations allowed the estimation of kinetic parameters (Figure 2). The diketide **2R,3R-8** was dehydrated by the DH to give exclusively the *E*-olefin product tigloyl SNAC **17**, but none of the other 2-methyl diketides showed any turn-over. Of the non-methylated diketides, only **3R-16** was a substrate, although much slower than **2R,3R-8**. The ability of the non-substrates **2S,3S-8** and the enantiomers of **13** to act as inhibitors of the DH was investigated. However, addition of each of these compounds to assays containing the substrate **2R,3R-8** showed no appreciable decrease in rate when added in mM concentrations (see ESI).

Despite having access to soluble protein we were unable to grow satisfactory crystals of the isolated DH domain. In lieu of other structural information we built a model of SQTKS DH based on the known crystal structures of DH proteins from other Type I systems reported in the literature. These form

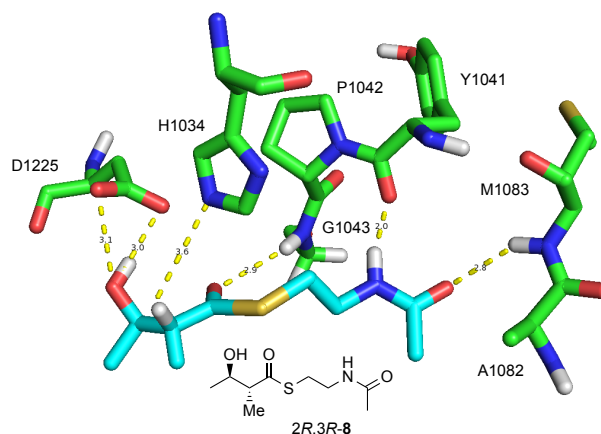
distinctive double hot-dog folds.<sup>17</sup> In particular the DH domain from CurF,<sup>18</sup> a modular type I PKS, formed an appropriate template for the assembly of a model by the SwissModel threading server.<sup>19</sup> Comparison of the results showed that the backbone atoms of the SQTCS DH model and CurF-DH had only 1.4 Å root mean square deviation (RMSD). Almost all of the observed deviation was concentrated on the periphery of the model structure, and examination of the conserved active site aspartic acid (D1225) and histidine (H1034) residues showed that these amino acids are located in the same positions in the CurF DH and the model (see ESI). In addition a highly conserved Y/FP motif (Y/F1041-P1042) is also preserved in the model.



**Figure 2.** Kinetic data for SQTCS DH reactions. **A**, raw time course data showing increase in tigloyl SNAC **17** concentration; **B**, comparison of DH reaction rate for the 4 diketide stereoisomers.

The diketide substrate **2R,3R-8** was then docked into the DH active site using a combination of manual positioning (PyMol)<sup>20</sup> and optimisation and energy minimisation using the YASARA force field.<sup>21</sup> For **2R,3R-8**, the docked model shows that the thioester carbonyl oxygen is within hydrogen bonding distance of the backbone NH of G1043 (2.9 Å), and aligns with the dipole of the helix between G1043 and M1055 of the DH model. The SNAC NH is positioned within hydrogen bonding distance of the backbone carbonyl of conserved Y1041 (2.0 Å); while the SNAC carbonyl is 2.8 Å from backbone NH of M1083. These interactions locate the SNAC in the entrance to the

reaction cavity and would presumably perform a similar role with the pantetheinyl-ACP-bound substrate in the native complex. The two methyl groups of **2R,3R-8** occupy open space and allow the  $\alpha$ -proton and  $\beta$ -hydroxyl to approach the conserved histidine and aspartate residues. The closest distance between the  $\alpha$ -proton and the nearest nitrogen of H1034 is 3.6 Å, while the distance between the  $\beta$ -hydroxyl and the oxygen of D1225 is 3.0 Å. The geometry of the interactions is consistent with a *syn* abstraction of water to give the observed *E*-2-methylbut-2-enoyl (tigloyl) SNAC product **17**. Attempts to dock the other stereoisomers of the diketide did not give satisfactory results.



**Figure 3.** Key modelled interactions between the substrate **2R,3R-7b** (cyan) and the DH (green).

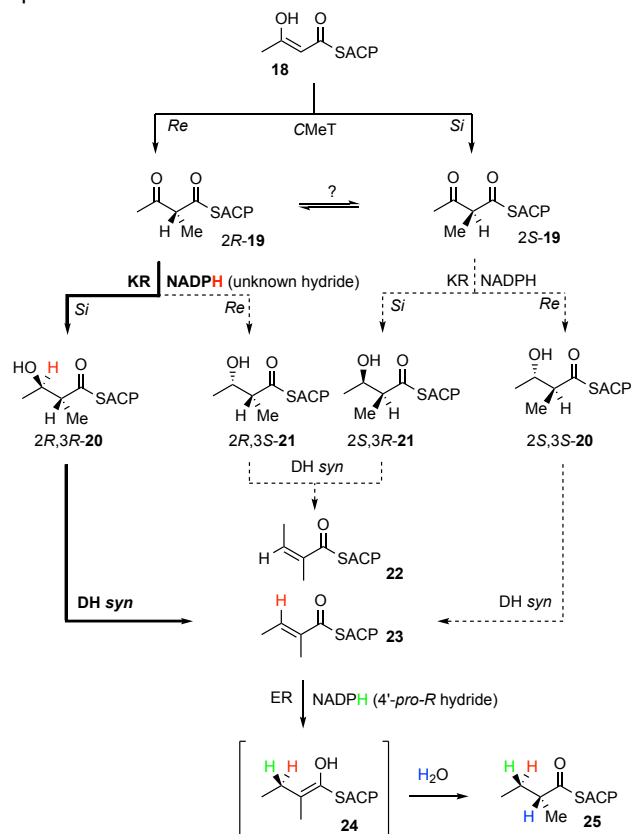
Our experiments report the first *in vitro* studies of the stereoselectivity of an isolated DH domain from an iterative Type I PKS. Using a kinetic assay we measured the  $K_M$  (4.5 mM) and  $k_{cat}$  values ( $0.063 \cdot \text{min}^{-1}$ ). While these values have little absolute meaning, they are comparable with values measured for other DH proteins. For example Aldrich, Smith and coworkers reported  $K_M$  values in the same range for a KR-DH didomain from module 2 of the pikromycin modular PKS (pikKR2-DH2) acting on triketide mimics, although their  $k_{cat}$  values are ten-fold higher.<sup>13</sup>

Only one 2-methylated diketide, **2R,3R-8**, is accepted as a substrate for SQTCS DH, with no dehydration activity observed for its enantiomer **2S,3S-8** or either of the *syn* diastereomers **13**. Since these stereoisomers show no measurable substrate or inhibition activity it seems unlikely that they can be bound at the DH active site, also supported by the failure to generate satisfactory docked models of these isomers. However the non-methylated diketide **3R-16** is a substrate.

In the active SQTCS the ACP-bound **2R,3R**-diketide **20** is created by reduction of a 3-oxo diketide **19** by the KR domain using NADPH as the cofactor (Scheme 3). Our results strongly suggest that the SQTCS KR releases *3R* substrates, and thus it must reduce the 3-oxo group of its substrate **19** by 3-*Si* addition of hydride. Since racemisation at the 2-position of the diketide is strongly disfavoured after reduction of the 3-oxo group, this observation also suggests that the KR accepts and releases *2R*-methylated diketides (e.g. **2R-19**, Scheme 3). However, because facile epimerisation of 2-methyl-3-oxo

substrates such as **19** is likely, it is not yet possible to infer the stereochemical preference of the C-MeT without further experiments. Our previous results have shown that the SQTCS ER domain can process both *Z* **22** and *E* **23** substrates. However since the DH can only provide *E*-diketides it appears that the ER's ability to accept *Z*-olefins is merely adventitious.<sup>10</sup>

In our earlier study of the stereoselectivity of the ER domain we showed that the stereochemical preferences at the  $\beta$ -carbon are identical for SQTCS ER and vFAS ER, in terms of both the cofactor itself (transfer of 4'-*pro-R* Hydrogen) and the substrate (addition of hydride to to the 3-*Re* face).<sup>10</sup> The results of this study also show that the SQTCS DH has exactly the same stereochemical selectivity as the vFAS DH which dehydrates 2*R*,3*R* substrates **20** to give *E*-products **23** by *syn* elimination.<sup>22</sup> Even though the SQTCS substrate is methylated at the 2-position, the 2*R* stereochemistry ensures that the 2-*pro-S* proton is removed during reaction. Our model structure shows that the 2*R*,3*R* substrate aligns with the active site residues such that *syn* elimination gives the observed *E*-product. The active site residues involved, H1034, D1225, Y1041 and P1042 are conserved between the SQTCS and vFAS sequences.



**Scheme 3.** Stereochemical course of KR, DH and ER domains of SQTCS.

Finally, the SQTCS KR domain also operates with the same stereochemical preference as the vFAS KR.<sup>22</sup> Although we have not yet been able to show which of the cofactor 4'-hydrides is transferred by KR, the reduction does occur at the 3-*Si* face of the substrate. Thus our studies show that SQTCS shares more than just sequence homology and domain organisation with

vFAS: its fundamental mechanisms for substrate reaction and stereoselectivity are also preserved and reinforce the idea that fungal hr-PKS and vFAS evolved from a common ancestor. Our current work focusses on determining the stereochemical preference of the CMeT domain and attempts at engineering SQTCS to rationally change its selectivity.

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